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New Ratiometric Fluorescent Calcium Indicators with Moderately Attenuated Binding Affinities

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Abstract—Mono-halogenated derivatives of the calcium indicators fura-2 and indo-1 were synthesized and their spectroscopic properties evaluated. Halogenation *ortho* or *para* to the bridging oxygen in the BAPTA nucleus had a more pronounced weakening effect on binding affinity than in the *meta* position in the fura derivatives. Two new excitation ratioable fluorescent calcium indicators, benzothiaza-1 and 2, were also synthesized. K_d values of 400 nM to 5.3 μM [Ca^{2+}] were observed in these families of new probes.

Fluorescent indicators of divalent metal ion concentrations such as Ca^{2+}, which were developed by Tsien and colleagues in the 1980's, have been of tremendous importance to researchers in a wide variety of biological science disciplines. Two of the most popular fluorescent calcium indicators are fura-2 and indo-1.[1] Both combine a fluorophore with a BAPTA (1,2-bis(2-aminophenoxy) ethane-*N,N,N,N*′-tetraacetic acid)[2] chelator and are optimally excited with UV light, and both exhibit ratiometric wavelength dependencies as a function of calcium concentration. The excitation maximum of fura-2 shifts from 362 to 335 nm as [Ca^{2+}] increases, while the emission wavelength of indo-1 shifts from 485 to 410 nm. This ratiometric property is very important, as it allows for internal calibration of [Ca^{2+}] and obviates the need for experimental corrections for photobleaching, sample thickness variability, dye concentration, etc. in intracellular systems.[3,4]

The binding affinity of fluorescent calcium probes such as fura-2 and indo-1 are most often expressed in terms of the dissociation constant K_d. K_d values for fura-2 and indo-1 are in the 140–230 nM range, which allows for accurate measurement of calcium concentrations close to these concentrations. However, when calcium concentrations are higher or are involved in fast transients, such as in cell excitatory states (intracellular) or when secreted (extracellular), these probes can saturate quickly and also partially act as buffers, resulting in inaccurate measurements.[5] For example, fura-2 has very limited sensitivity to [Ca^{2+}] above 1 μM.[6] Molecules like mag-fura-2,[7] in which the BAPTA portion is truncated into a lower affinity chelator, have an attenuated affinity for calcium but are quite sensitive to magnesium. London et al. began to address this selectivity problem by synthesizing fura-FF and indo-FF.[8,9] These difluorinated BAPTA-based low affinity probes have found some utility, for example being used to quantify high calcium concentrations in endoplasmic reticulum[10,11] and cytoplasm.[12,13] However, these probes' high calcium K_d values (30–50 μM, two orders of magnitude higher than the parent probes) make them relatively insensitive to [Ca^{2+}] in the 1–5 μM range. Our aim in the present study was to find fura-2 and indo-1 analogues with binding affinities only about one order of magnitude less than the parent probes, filling a needed gap in the bioscientist's toolbox for optical calcium concentration measurements. We achieved this aim by exploring the effect of single halogen atom attachment to various positions on the BAPTA chelator part of the fluorescent probe molecules, and also by synthesizing a new fluorescent calcium indicator. The syntheses and spectroscopic properties are reported herein.

The synthetic strategy for fura-4F, fura-5F, fura-5Cl, and fura-6F (**5a–d**) derives from that originally reported by Grynkeiwicz et al.,[1] beginning with an appropriately halogenated 2-nitrophenol (**2**); this was also the strategy employed in the synthesis of fura-FF[8,9] (Scheme 1).

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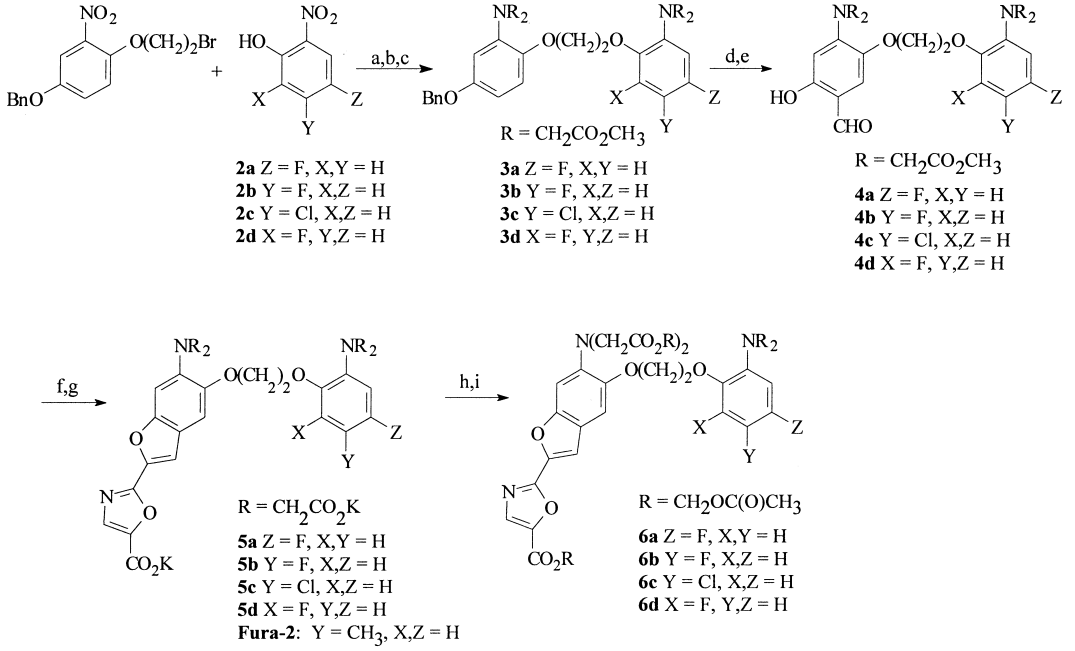
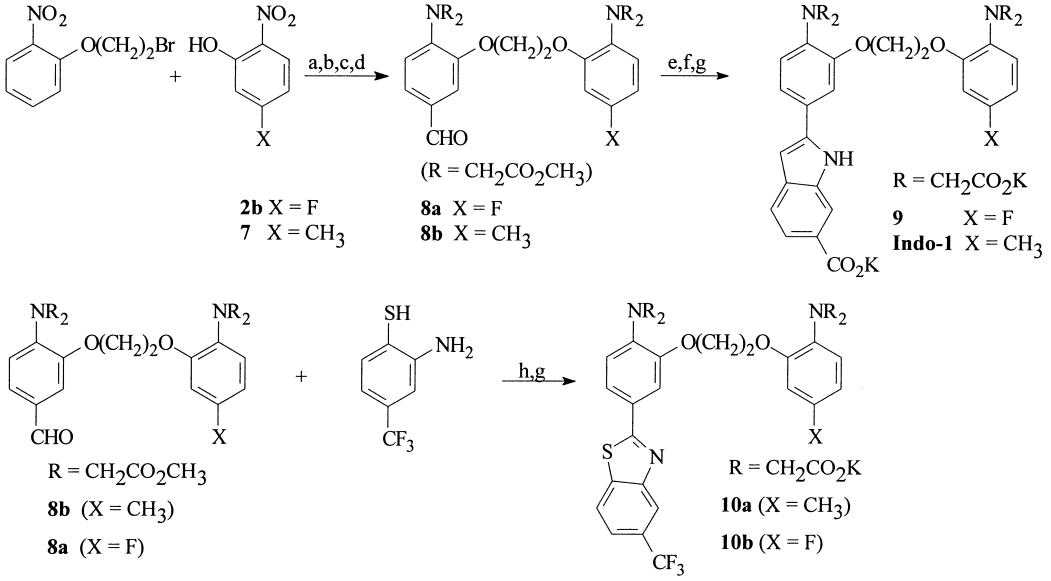
Indo-5F (**9**) was obtained beginning with 5-fluoro-2-nitrophenol and 2-nitrophenol. Construction of the BAPTA nucleus went as described above, followed by formylation, to give key intermediate **8a**. Wittig reaction and reductive cyclization, as described for indo-1,[1] gave the desired indicator after saponification of the esters (Scheme 2). Condensation of 5-formyl-5′-methyl-BAPTA methyl ester[1] (**8b**) and **8a** with 5-trifluoromethyl-2-aminothiophenol in DMSO gave the novel dyes benzothiaza-1 (**10a**) and-2 (**10b**), respectively, after ester saponification (Scheme 2).

All of the probes were purified and characterized in their potassium salt forms.[14] From this, they were convertable into their cell permeant acetoxymethyl (AM) ester[15] forms (**6**) by conversion to the free acids, followed by alkylation with bromomethyl acetate in DMF, mediated by diisopropylethylamine.

In the monohalogenated fura series (**5a–d**), the halogen atom exerted a larger effect on calcium binding affinity when located *ortho* or *para* to the bridging oxygen atom of the BAPTA nucleus, as opposed to when *para* to the



Scheme 1. Reagents: (a) K_2CO_3, DMF; (b) H_2, Pd/C; (c) $BrCH_2CO_2CH_3$, DMF; (d) $POCl_3$, DMF; (e) H_2, Pt/C; (f) 5-chloromethyloxazole-2-carboxylic acid, ethyl ester, K_2CO_3, DMF; (g) KOH(aq), MeOH/dioxane; (h) HCl; (i) $BrCH_2OC(O)CH_3$, DIEA, DMF.



Scheme 2. Reagents: (a) K_2CO_3, DMF; (b) H_2, Pd/C; (c) $BrCH_2CO_2CH_3$, DMF; (d) $POCl_3$ DMF; (e) 4-Methoxycarbonyl-2-nitrobenzyl-α-triphenylphosphonium bromide, K_2CO_3, DMF; (f) $P(OEt)_3$; (g) KOH(aq), MeOH/dioxane; (h) DMSO.

Table 1. Binding affinity and wavelengths

Compound	K_d (Ca^{2+})	Exc λ_{max} (low Ca^{2+})	Exc λ_{max} (high Ca^{2+})	Em λ_{max}	Ref
Fura-2	140 nM	364 nm	338 nm	512 nm	1
Fura-4F (**5a**)	770 nM	371 nm	339 nm	510 nm	
Fura-5F (**5b**)	400 nM	367 nm	339 nm	511 nm	
Fura-5Cl (**5c**)	400 nM	366 nm	337 nm	511 nm	
Fura-6F (**5d**)	5.3 μM	367 nm	337 nm	510 nm	
Benzothiaza-1 (**10a**)	660 nM	368 nm	333 nm	470 nm	
Benzothiaza-2 (**10b**)	1.4 μM	367 nm	331 nm	470 nm	
Indo-1	230 nM	349 nm	331 nm	485/410 nm[a]	1
Indo-5F (**9**)	470 nM	346 nm	330 nm	478/401 nm[a]	

[a]Emission maxima in low/high [Ca^{2+}]; excitation at 334 nm.

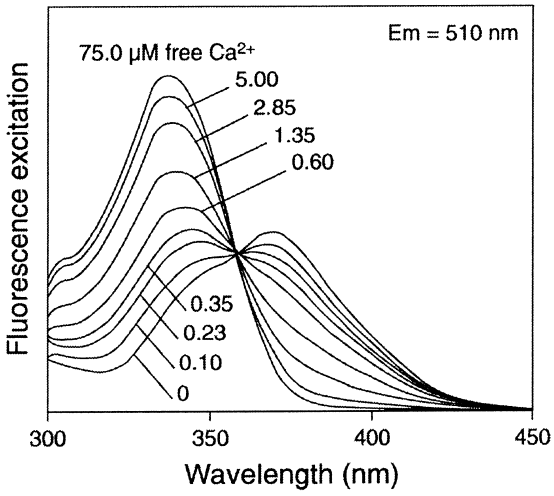


Figure 1. Fluorescence excitation spectra of **5a**.

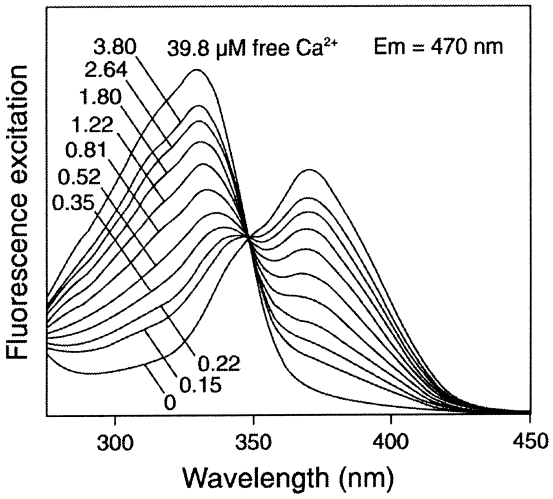


Figure 2. Fluorescence excitation spectra of **10a**.

BAPTA nitrogen atom (Table 1). In the 5′-halo-fura molecules **5b–c**, chlorine or fluorine had essentially the same effect. All of the monohalogenated fura derivatives had fluorescence quantum yields similar to fura-2 in buffer, with little change in wavelength maxima.

The modest effect of 5′-halogenation was also observed for indo-5F (**9**), which has a 2-fold drop in binding affinity relative to the 5′-methyl analogue, aka indo-1.

The benzothiaza probes **10a–b** exhibit the same type of excitation ratiometric behavior with changing [Ca^{2+}] as fura-2 does, at the same wavelengths (Fig. 2). Their straightforward synthesis allows for easy structural modification. However, the emission wavelengths of the benzothiaza dyes are about 40 nm shorter than fura-2. This property may allow for dual imaging of benzothiaza and fluorescein-based probes, since the fluorescence emissions can be measured distinct from each other. Such an experiment is not possible with fura derivatives, because the emission (510 nm) coincides with that of fluoresceins (510–520 nm). Benzothiaza-2 (**10b**) achieved the goal of about 10-fold lower binding affinity, compared with fura-2. However, the quantum yields of both benzothioaza-1 and-2 are somewhat lower, i.e. about 25% that of fura-based probes. Nevertheless, we obtained successful loading of 3T3 fibroblasts, RBL mast cells and BC3H-1 myocytes using incubation at 37 °C for 30 min with 1–5 μM (**10a**) as its tetra-AM ester, imaged via fluorescence microscopy using standard fura-2 optics. We also observed the expected shift in excitation maxima from 370 to 330 nm upon addition of the calcium ionophore ionomycin to the extracellular medium (data not shown). Also, BHK cells have been loaded with **10a** as its tetra-AM ester for [Ca^{2+}] measurement by two-photon laser scanning microscopy.[4]

Acknowledgements

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14. For example, for **5d**: ^{1}H NMR (300 MHz, D_2O) δ 7.65 (s, 1H), 7.55 (s, 1H), 7.36 (s, 1H), 7.08 (s, 1H), 6.97 (dd, $J=14$, 6.6 Hz, 1H), 6.67 (t, $J=9.6$ Hz), 6.50 (d, $J=8.4$ Hz, 1H), 4.35 (br s, 4H), 3.99 (s, 4H), 3.93 (s, 4H); ^{19}F NMR (D_2O) ϕ 67.0 (referenced to CF_3–toluene); HPLC 99% pure (254 nm, C18 4.6×250 mm column, 5–95 CH_3CN/0.1% TFA for 30 min, retention time 19.9 min); E 25,000 cm^{-1} M^{-1} (10 μM EGTA/10 μM MOPS, pH 7.2).
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